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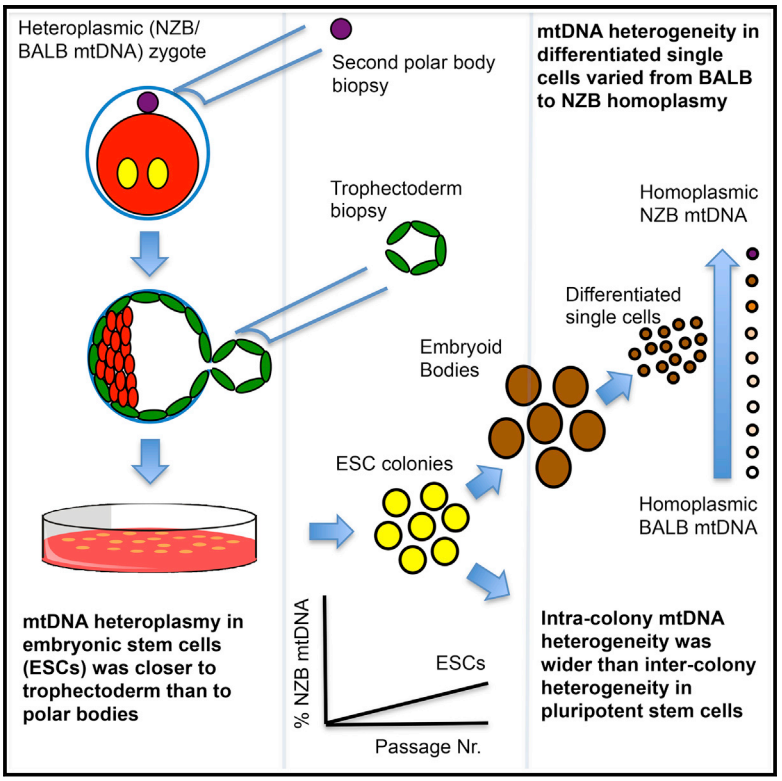
Cellular Heterogeneity in the Level of mtDNA Heteroplasmy in Mouse Embryonic Stem Cells

Graphical Abstract



Highlights

- mtDNA heteroplasmy is compared in ESCs, trophectoderm (TE), and second polar bodies (PB2)
- mtDNA heteroplasmy in ESCs is more closely associated with TE cells than PB2
- Cellular heterogeneity of homoplasmic mtDNA haplotypes occurs at the single-cell level
- The level of mtDNA heteroplasmy increases with progressive passage numbers

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In Brief

Neupane et al. show that, in comparison to second polar bodies, mtDNA heteroplasmy in trophectoderm (TE) cells is more closely correlated with the corresponding ESCs, indicating that TE biopsy is preferable to polar body biopsies for pre-implantation genetic diagnosis in mtDNA disorders. Wider variability in mtDNA heteroplasmy in stem cells could explain the existence of tissues with different heteroplasmic loads in individuals with heteroplasmic mtDNA conditions.

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Cellular Heterogeneity in the Level of mtDNA Heteroplasmy in Mouse Embryonic Stem Cells

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SUMMARY

Variation in the level of mtDNA heteroplasmy in adult tissues is commonly seen in patients with a mixture of wild-type and mutant mtDNA. A mixture of different mtDNA variants may influence such variation and cause mtDNA segregation bias. We analyzed cellular heterogeneity in embryonic stem cells (ESCs) derived from a polymorphic mouse model containing NZB and BALB mtDNA genotypes. In ESCs, inter-colony heterogeneity varied up to 61%, whereas intra-colony heterogeneity varied up to 100%. Three out of five cell lines displayed nearly homoplasmic BALB and NZB mtDNA haplotypes in differentiated single cells. The proportion of NZB mtDNA genotype increased with progressive passaging (0.39%; p = 0.002). These results demonstrate the bimodal segregation of mtDNA haplotypes, indicating the occurrence of tissues with variable levels of heteroplasmies in individuals with mtDNA mutations. Furthermore, proliferation of one mtDNA genotype over another may pose the risk of accumulating mutant mtDNAs during subsequent cell divisions.

INTRODUCTION

Normally, all copies of mtDNA in a cell are identical ("homoplasmy"). However, more than one type of mtDNA variant may exist within a cell ("heteroplasmy"). Mutation, deletion, or depletion of mtDNA results in mtDNA disorders, which can be lethal (Wallace, 1999). In cases of mtDNA mutation disorders, a mixture of wild-type (WT) and mutated mtDNA copies are present. Intercellular variation in the level of mtDNA heteroplasmy is frequently seen in individuals with heteroplasmic mtDNA mutations. The mtDNA bottleneck followed by random genetic drift is thought to be responsible for the unbalanced segregation of heteroplasmic mtDNAs in the subsequent cell divisions (Jenuth et al., 1996). Unlike nuclear DNA, mtDNAs are inherited uniquely from the maternal line of origin (Al Rawi et al., 2011; Birky, 2001). During oogenesis in the maternal germline, there is a 1,000-fold increase in mtDNA copy numbers from the primordial germ cells (PGCs) to the mature metaphase II (MII) oocytes (Cao et al., 2007, 2009; Cree et al., 2008; Monnot et al., 2013; Pikó and Taylor, 1987; Wai et al., 2008). If mtDNA is present in a heteroplasmic form in the PGCs, then either of the mtDNA variants can be selected for or against (St John, 2014), leading to intercellular variability. In the absence of a therapeutic remedy, pre-implantation genetic diagnosis (PGD) and germline genome transfer are the only possible approaches to avoid the transmission of heritable mtDNA disorders. However, in both cases, the potential risk of mtDNA carryover to the offspring may result in a heteroplasmic condition, resulting in the unbalanced segregation of mtDNA variants.

Previous studies in heteroplasmic mouse models have shown that one mtDNA haplotype can proliferate faster than the other, both in mitotic (Jenuth et al., 1997; Sharpley et al., 2012) and post-mitotic tissues (Burgstaller et al., 2014). However, the reason behind the mtDNA segregation bias is unknown. In order to elucidate whether polymorphic mtDNA haplotypes influence this segregation bias, we analyzed embryonic stem cells (ESCs) and their differentiated counterparts in a heteroplasmic mouse model. The heteroplasmic BALB/OlaHsd mouse model was generated as described previously (Jenuth et al., 1996) (kindly gifted by Dr. Brendan J. Battersby from FinMIT Academy of Finland Centre of Excellence, University of Helsinki). These mice were polymorphic for two mtDNA haplotypes coming from BALB/cByJ and NZB/OlaHsd mouse models. These two mtDNA haplotypes differed by 101 nt (~15 amino acid substitutions) (Dean et al., 2003). Using these mouse models, we analyzed cellular heterogeneity in the level mtDNA heteroplasmy in pluripotent and differentiated stem cells, both in colonies and at the single-cell level. Furthermore, to test the reliability of the samples for biopsy during PGD, we also compared the level of mtDNA heteroplasmy in ESCs with their corresponding second polar bodies (PB2) and trophectoderm (TE) cells originating from the same embryos (Figure 1).

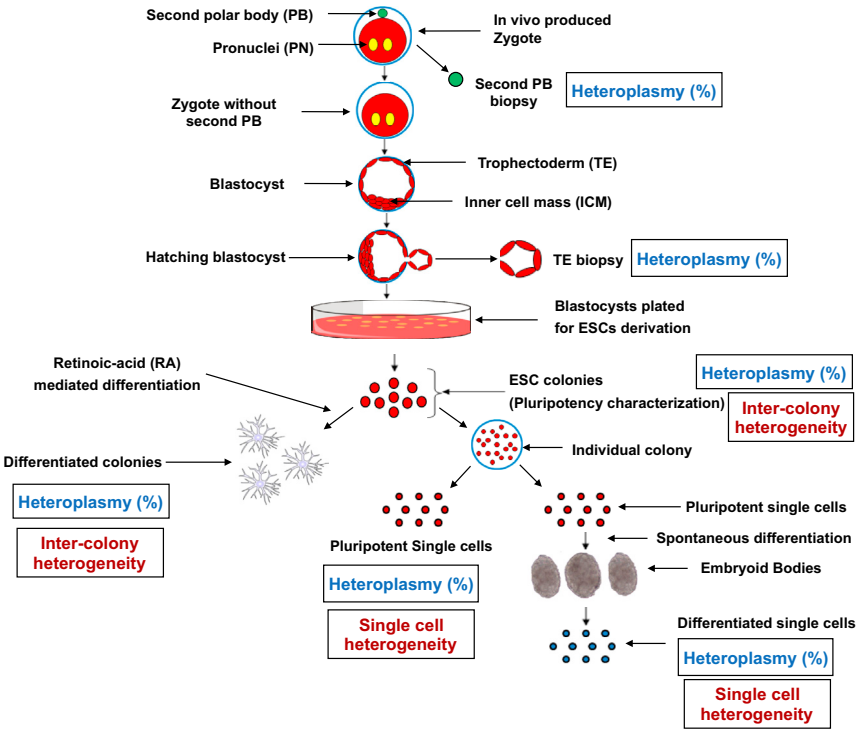

Figure 1. Schematic Diagram Showing the Study Design of Cellular Heterogeneity in the Level of mtDNA Heteroplasmy in Mouse Embryonic Stem Cells and Their Relation with the Corresponding Founder Embryos

Second polar bodies (PBs) were biopsied from in-vivo-produced zygotes to measure the level of mtDNA heteroplasmy. Zygotes (devoid of second PBs) were cultured until blastocyst stage from which trophectoderm (TE) cells were biopsied for measuring mtDNA heteroplasmy. Whole blastocysts (subjected to P2B and TE biopsy) were plated to derive embryonic stem cells (ESCs). The level of mtDNA heteroplasmy was measured in pluripotent ESC colonies on different passages during culture to analyze inter-colony heterogeneity (p8–p40). Inter-colony heterogeneity was also measured in retinoic-acid-mediated differentiated cells (p30). To analyze intra-colony heterogeneity, single cells from an individual pluripotent ESC colony were recovered (p40) and the remaining cells from the same colony were differentiated spontaneously as embryoid bodies (EBs) for 15 days. The level of heteroplasmy was then measured in pluripotent and differentiated single cells. Characterization of pluripotent stem cells is shown in Figure S1.

RESULTS

TE Cells Are Informative of the Level of mtDNA Heteroplasmy in ESCs

The survival rate of the embryos, following two biopsies, was 100% (n = 14); PB2 biopsy was done at the zygote stage and TE biopsy on same embryo at the blastocyst stage. Heteroplasmic ESC lines were derived from eight embryos (~57%), five of which were used for further analysis. First, we investigated the association between the level of mtDNA heteroplasmy in the TE cells and that in the corresponding PB2, and we found no evidence of association between them (R = 0.14; p = 0.64) (Table S2). As TE cells have been shown to represent the inner cell mass (ICM) (Treff et al., 2012) and whole blastocysts (Heindryckx et al., 2014; Neupane et al., 2014b), the poor correlation between the PB2 and their corresponding TE cells confirmed that polar bodies were not reliable samples for biopsy during PGD to avoid the transmission of pathogenic mitochondrial diseases. In terms of coefficient of determination (R^2), 4.7% of the variability in ESCs could be attributed to a difference in PB2, whereas 19.1% was due to the difference in TE cells, indicating that the level of mtDNA heteroplasmy in TE cells was more closely associated with that in ESCs than in PB2 (Tables S1 and S2).

mtDNA Heteroplasmy Increases with Progressive Passaging

Next, we analyzed the level of mtDNA heteroplasmy in undifferentiated ESC colonies at different passages (n = 137) (Figure 2; Table S1) and also in differentiated colonies (p30; n = 42) (Table S1). Heterogeneity in the level of mtDNA heteroplasmy was compared between individual colonies (inter-colony heterogeneity) within the same line in five cell lines with variable heteroplasmic loads. For measuring inter-colony heterogeneity, five to ten individual colonies were randomly selected from each line. Inter-colony heterogeneity in the proportion of NZB mtDNA haplotype in pluripotent ESC colonies ranged from 12.2% to 21.5% in line 1, 14.0% to 49.8% in line 2, 14.7% to 54.5% in line 3, 19.3% to 61% in line 4, and 13.1% to 39.9% in line 5 (Table S1). Interestingly, the proportion of mean NZB mtDNAs increased from 65.8% to 88.7% in line 1, 45.8% to 54.9% in line 2, 47.0% to 64.1% in line 3, 79.2% to 86.9% in line 4, and 90.1% to 92.4% in line 5 (Figure 2; Table S1). The statistical analysis revealed a strong evidence of an increase in the NZB mtDNA haplotype with each passage (mean increase per passage 0.39%, p = 0.002) (Figure 2; Table S1).

In order to investigate if the ESCs exhibit segregation bias after differentiation, we analyzed the level of mtDNA heteroplasmy in differentiated colonies (RA-mediated differentiation on p30) from each ESC line. In differentiated cells, inter-colony heterogeneity varied by 7.6% in line 1, 45.3% in line 2, 27.4% in line 3, 1.5% in line 4, and 45.2% in line 5 (Table S1). However, no evidence of the difference in the level of heteroplasmy between differentiated and undifferentiated cells (mean difference −0.72, p = 0.74) in the ESC colonies was observed.

Single-Cell Heterogeneity Displayed a Homoplasmic State for mtDNA Haplotypes

Given that each ESC colony comprises hundreds of single cells, we aimed to investigate if individual cells within a colony exhibit difference in the proportion of mtDNA haplotypes. For this, heterogeneity at the single-cell level in pluripotent (n = 79) and differentiated (n = 107) ESCs was studied. We analyzed 15–22 single

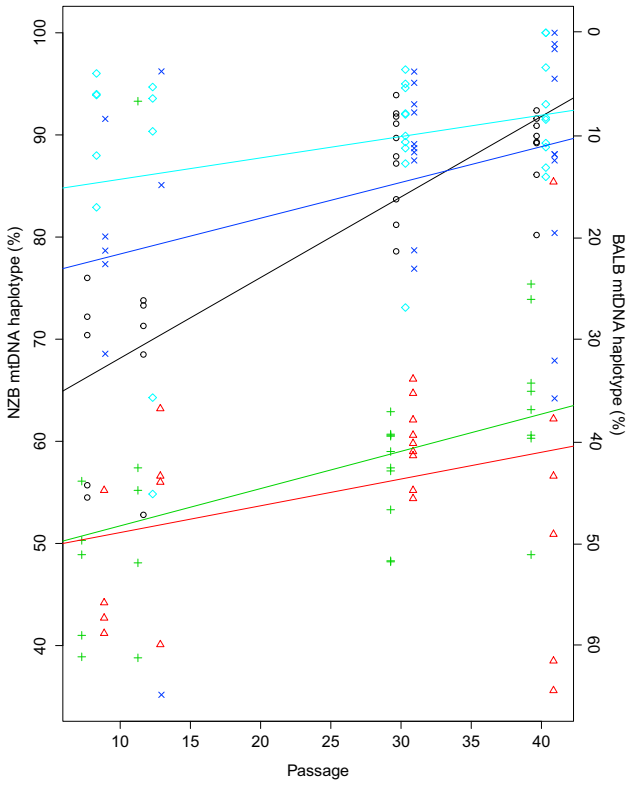

Figure 2. Variation in the Level of mtDNA Haplotypes as a Function of Passage, Shown in Five Different Cell Lines

Level of mtDNA heteroplasmy (% of NZB mtDNA haplotype) was measured in five to ten pluripotent ESC colonies from cell lines 1 (black), 2 (red), 3 (green), 4 (blue), and 5 (cyan) on passages 8, 12, 30, and 40. The mean NZB haplotype proportion increased from 65.8% to 88.7% in line 1, 45.8% to 54.9% in line 2, 47.0% to 64.1% in line 3, 79.2% to 86.9% in line 4, and 90.1% to 92.4% in line 5. The proportion of NZB mtDNA haplotype in the pluripotent ESCs increased by 0.39% per passage during culture ($p = 0.002$). Data are presented in Table S1. A schematic diagram showing the heteroplasmic load measurement is shown in Figure S2.

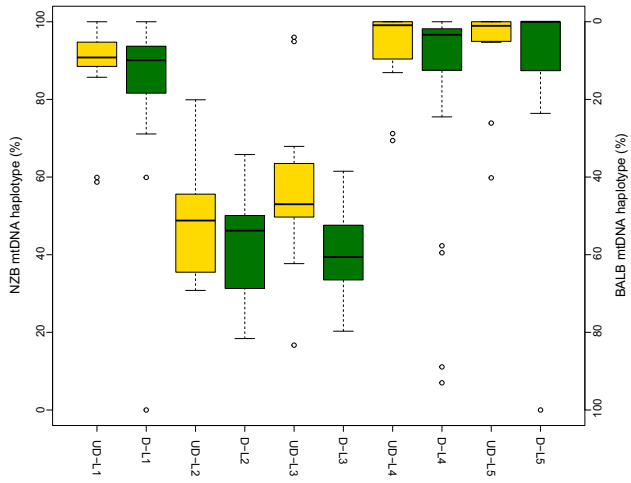

Figure 3. Boxplots Showing Variation in the Proportion of NZB and BALB mtDNA Haplotypes in Undifferentiated and Differentiated Embryonic Stem Cells in Five Cell Lines

Single-cell analysis displayed homoplasmic states for mtDNA haplotypes in three cell lines (L1, L4, and L5) before and/or after differentiation. In undifferentiated single cells (yellow), homoplasmic NZB mtDNA was observed in cell lines 1, 4, and 5. In differentiated single cells (green), homoplasmic states for both the mtDNA haplotypes were observed in cell lines 1 and 5, whereas a homoplasmic state for NZB mtDNA was observed in cell line 4 (Table S1). A standard curve showing the detection limit and the efficiency of heteroplasmic load measurement is shown in Figure S3 (Table S3). UD, undifferentiated; D, differentiated; L, cell line.

cells randomly selected from one colony from each cell line, before and after differentiation, originating from the same colony. The proportion of NZB mtDNA haplotype in pluripotent single cells varied from 59.9% to 100% in line 1, 30.8% to 79.9% in line 2, 16.7% to 96% in line 3, 69.4% to 100% in line 4, and 59.8% to 100% in line 5 (Table S1). In pluripotent single cells, the level of heteroplasmy varied by ~36% on average (Figure 3; Table S1).

We further investigated whether the difference in the proportion of NZB and BALB mtDNA haplotypes observed at the single-cell level in the pluripotent state varied after differentiation. For this purpose, we analyzed the proportion of mtDNA haplotypes present in single ESCs after spontaneous differentiation by forming embryoid bodies (EBs). Much wider variation in the proportion of mtDNA haplotypes was observed in differentiated single cells, demonstrating homoplasmic haplotypes for both NZB and BALB mtDNA (Figure 3; Table S1). The statistical analysis showed a significant difference in the level of mtDNA heteroplasmy between pluripotent and differentiated single cells (mean difference = −8.5%; $p = 0.0014$). The proportion of NZB mtDNA haplotype in differentiated single cells varied from 0% to 100% in line 1, 18.4% to 65.8% in line 2, 20.3% to 61.5% in line 3, 7% to 100% in line 4, and 0% to 100% in line 5 (Figure 3; Table S1). When these single cells were grouped into different categories as <30%, <30%–70%, and >70%, more than half (53%) of the analyzed single cells showed segregation toward NZB haplotype (Table S1). Slightly more than one-third (34%) of the cells showed bidirectional segregation, and nearly one-eighth (13%) of the cells showed affinity toward the BALB mtDNA haplotype. However, no statistical difference was observed in mtDNA heteroplasmy levels between single cells and their corresponding colonies obtained from the same passage (p40) ($p = 0.21$) (Table S1).

DISCUSSION

In this study, first we compared the level of mtDNA heteroplasmy in PB2, TE cells, and their corresponding ESCs in heteroplasmic mouse models. We then analyzed cellular heterogeneity at the stem cell level, both in pluripotent and differentiated states. Paull et al. (2013) have investigated the mtDNA heterogeneity in pluripotent and differentiated stem cells to detect the re-emergence of heteroplasmic mtDNA after germline nuclear transfer, if any, in humans. While the transmitted mtDNA was detected at very low levels (<1%) initially, no detectable mtDNA heteroplasmy was observed in the isolated stem cell lines, even after extensive passaging or differentiation (Paull et al., 2013). They investigated heterogeneity in stem cell colonies derived from single cells and

also in each of the germ layers after differentiation, unlike our study, where we assessed the mtDNA heteroplasmy in single cells from pluripotent stem cell colonies and EBs in a heteroplasmic mouse model.

In line with the current study where we observed a poor correlation between PB2 and TE cells, previous studies have also reported a poor correlation between PBs and oocytes or blastomeres (Gigarel et al., 2011; Vandewoestyne et al., 2012) and a strong correlation between TE cells and inner cell mass (Treff et al., 2012) or blastocysts (Heindryckx et al., 2014). The poor correlation between PB2 and TE cells could be due to at least two factors: (1) random genetic drift may lead to unbalanced distribution of mtDNA haplotypes during successive cell divisions in the embryos (Jenuth et al., 1996), and (2) biased inheritance of mtDNA haplotypes during asymmetric cell division in the preimplantation embryos may cause variation in the proportion of mtDNA haplotypes in the daughter cells (Dalton and Carroll, 2013).

Unlike previous studies that have shown unidirectional loss of mtDNA heteroplasmy in the pluripotent stem cells (PSCs) during in vitro culture (Cherry et al., 2013; Folmes et al., 2013), we did not observe such a phenomenon. The bimodal variation in the level of mtDNA heteroplasmy, observed in the current study, to either the BALB homoplasmic state or NZB homoplasmic state at the single-cell level could be due to asymmetric mtDNA transmission during subsequent cell divisions. In line with our study, Hämäläinen et al. (2013) demonstrated bidirectional segregation of mtDNAs in induced PSCs toward WT or mutant homoplasmy, indicating no clear selection for or against mutant m.3243A > G mtDNA upon reprogramming (Hämäläinen et al., 2013).

Tissue-specific variability with bimodal segregation of polymorphic mtDNA variants was reported in the earlier studies in heteroplasmic mouse models (Jenuth et al., 1997; Sharpley et al., 2012). However, directional loss of mtDNA variants was seen in the mitotic tissues in these mice, resulting in tissue-specific variability. A recent study in heteroplasmic mouse models demonstrated mtDNA segregation bias in post-mitotic tissues within the same subspecies and showed a prenatal segregation pattern (Burgstaller et al., 2014). They showed that mtDNA segregation bias could occur in polymorphic mouse models with an admixture of mtDNA haplotypes. After analyzing intermediate to high heteroplasmic ESC lines in the current study, our results demonstrated the proliferation of the NZB mtDNA haplotype during progressive passage of pluripotent ESCs, irrespective of the original mtDNA proportion. The mechanism by which heteroplasmic mtDNA segregation bias occurs is unknown. It might be possible that one mtDNA haplotype is selected against another (Burgstaller et al., 2014); however, there is no proof to support this phenomenon, and it could vary in different models.

A recent study by our group demonstrated the divergent mutant load distribution in peripheral blood mononuclear cells (PBMCs), ranging from homoplasmic WT (0%) to nearly homoplasmic mutant (~95%) mtDNA variants in a patient with an m.3243A > G (MELAS) point mutation (Vandewoestyne et al., 2011). Implying such tremendous variability at the single-cell level in ESCs and PBMCs, we speculate that embryos with variable mutation loads could be screened during PGD in individuals with heteroplasmic mtDNA mutations. This could provide an opportunity for fertility clinics to search for mutation-free embryos to prevent the transmission of pathogenic mtDNA mutations, as was reported in our previous study (Heindryckx et al., 2014). From an ethical perspective, if an mtDNA mutation could be managed by a PGD approach by selecting and transferring mutation-free embryos (or those with undetectable mutant loads), it would avoid the use of donor oocytes/embryos, thereby avoiding the involvement of a third parent, which is required for germline nuclear transfer techniques (Craven et al., 2010; Neupane et al., 2014a; Tachibana et al., 2013).

The heterogeneous distribution of mtDNA heteroplasmy in single cells is in concordance with the previous studies showing variability in mtDNA heteroplasmy in organs of adult mice toward either of the mtDNA genotypes, indicating that the segregation of polymorphic mtDNAs may not be neutral (Burgstaller et al., 2014; Jenuth et al., 1997; Sharpley et al., 2012). However, a striking difference may exist between germline and somatic cell mtDNA heteroplasmy segregation as reported by Sharpley and colleagues (2012). Furthermore, passage-associated increase in NZB mtDNA haplotype during in vitro cell culture was irrespective of the original mtDNA proportion, showing the unbalanced proliferation of a heteroplasmic mtDNA genotype. Proliferation of mtDNA genotypes has been associated with the difference in mtDNA genotypes, with proportional increment corresponding to distant genetic haplotypes (Burgstaller et al., 2014). Furthermore, proliferation of one mtDNA genotype could be linked with the selective advantage over another in some tissues, resulting in tissue-specific segregation (Takeda et al., 2000). However, such a selection might be influenced by the in vivo or in vitro state of the cells. Battersby and Shoubridge (2001) reported the selective advantage of NZB mtDNAs over BALB mtDNAs in mouse liver cells in vivo. However, isolation and in vitro culture of hepatocytes from the livers of same heteroplasmic mice reversed the segregation direction, enriching BALB mtDNAs (Battersby and Shoubridge, 2001), which indicates that growth medium might influence the segregation bias. An increase in level of NZB mtDNA in the current study might be due to the influence of in vitro culture, which needs further investigation in vivo. Nevertheless, the mechanism and direction of segregation might be due not to a difference in replication rate between two genotypes but to the maintenance of mitochondrial genome or mitochondrial turnover (Battersby and Shoubridge, 2001; Wallace and Chalkia, 2013).

The present study points out the risk of mtDNA segregation bias at the stem-cell level, which may occur if the embryo selected for PGD is not mutation-free or if the mtDNA carryover after meiotic nuclear transfer is not undetectable. However, results from animal models should be cautiously extrapolated to humans, since the species-specific difference in mtDNA segregation between mouse models and humans cannot be ignored. Moreover, polymorphic mtDNA may segregate differently than mutant mtDNA. Therefore, to avoid the concern of limited measurements and animal models, more studies should be conducted with bigger sample sizes to confirm these results in human.

We report cellular heterogeneity in the ESCs in a mouse model with different mtDNA haplotypes, along with the relation between ESCs and their founder embryos (PB2 and TE). Selective proliferation of one mtDNA haplotype over another may result in

the potential amplification of mutant mtDNA with aging, as discussed recently (Ye et al., 2014). Furthermore, wider heterogeneity observed in differentiated single cells corresponds with the tissue-specific variability in the levels of heteroplasmies in individuals with mtDNA mutations. However, further studies are required to explore the germ layer specific segregation of mtDNA.

EXPERIMENTAL PROCEDURES

Embryo Collection and Micromanipulation

In-vivo-fertilized zygotes were collected from superovulated heteroplasmic BALB/cOlaHsd female mice, which contained the mtDNA mixture of BALB/cByJ and NZB/OlaHsd strains. PB2 were biopsied from zygotes, and the remaining zygote was cultured in potassium simplex oxidized medium (KSOM) until the morula stage and subsequently in Cook Blastocyst medium until the blastocyst stage. TE cells were biopsied from hatching blastocysts, and the remaining blastocyst was used for ESC derivation (Figure 1; Figure S1).

ESC Derivation and Differentiation

Mouse ESCs were derived as described elsewhere (Ghimire et al., 2015). For inducing differentiation, ESCs were cultured in N2B27 medium containing 1 μM retinoic acid (RA) for 7 days (without 2i and leukemia inhibitory factor [LIF]). For spontaneous differentiation, cells were plated in low-attachment 24-well plates in N2B27 medium (without 2i and LIF) for 15 days to form EBs.

Polymerase Chain Reaction Restriction Fragment Length Polymorphism

Isolated TE cells and ESCs were transferred to separately labeled microcentrifuge tubes (Westburg) containing 10 μl PicoPure DNA extraction buffer with proteinase K (PicoPure DNA extraction kit, Arcturus). All samples were incubated at 65°C for 3 hr, centrifuged briefly, and heated at 95°C for 10 min to inactivate proteinase K. PCR restriction fragment length polymorphism (RFLP) was used to measure the level of mtDNA heteroplasmy in the different stages of embryos and ESCs (Figures 1 and S2). Nested PCR was performed to amplify mtDNAs from single ESCs. First PCR amplification was performed using the forward primer NPMT9 (5′-ATATCAATTTACCAGAACTCTAC-3′), annealing the mitochondrial genome from nucleotides 3,493 to 3,515, and the reverse primer NPMT10 (5′-CCTAGTTGTTTATAGTTGAGTAC-3′), annealing the mitochondrial genome from nucleotides 4,157 to 4,135. Second PCR amplification was performed using the 6-fluorescein amidite (6-FAM)-labeled forward primer MT9 (5′-GAGCATCTTATCCACGCTTCC-3′), annealing the mitochondrial genome from nucleotides 3,571 to 3,591, and the reverse primer MT10 (5′-CTGCTTCAGTTGATCGTGGGT-3′) (labeled with (1-naphthyl) ethylenediamine dihydrochloride [NED]), annealing the mitochondrial genome from nucleotides 4,059 to 4,079 (Tables S4 and S6).

After restriction digestion, heteroplasmic amplicons were cleaved into a 6-FAM-labeled 121-bp fragment (exclusively coming from BALB mtDNA), an NED-labeled 170-bp fragment (common for NZB and BALB mtDNA), and a 6-FAM-labeled 339-bp fragment (exclusively coming from NZB mtDNA).

Capillary Electrophoresis and Calculation of Heteroplasmic Load

The amplified mtDNA fragments were analyzed by capillary electrophoresis using an ABI 3130 Genetic Analyzer (Applied Biosystems). The heteroplasmic load (proportion of NZB mtDNA) was determined by calculating the ratio of the area of the 6-FAM labeled 339-bp fragment and the sum of the areas of the 6-FAM 339-bp fragment and the 6-FAM 121-bp fragment, multiplied by 100 (Figure S2). Heteroplasmic load below 2% was considered undetectable. Relation between input and output heteroplasmy has been shown in Figure S3 (Table S3).

Statistical Analysis

The analysis of the embryonic stem cells was based on linear mixed models to account for (random) line effects. The multivariate model included linear effects of differences between differentiated and undifferentiated cells. It moreover allowed for the variability in the level of heteroplasmy to change over passages. Marginal residual plots confirmed the adequacy of the final models. Statistical analyses were performed in RStudio version 0.97.320 and Microsoft Excel. p values below 0.05 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.10.019.

AUTHOR CONTRIBUTIONS

J.N. conceived, designed, and performed experiments, analyzed the data, and wrote the manuscript. S.G. derived and differentiated ESCs, performed immunostaining and qRT-PCR, and wrote the manuscript. Y.L. helped in animal handling and data collection. T.D. and M.V. supervised the experiment and reviewed the manuscript. T.D. conceived and helped in optimizing single-cell PCR experiments. S.V. interpreted the data, derived a statistical model, and wrote the manuscript. J.G. and R.V.C. reviewed the manuscript and provided critical suggestions. B.H., D.D., and P.D.S conceived and supervised the experiment and reviewed the manuscript.

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